Universidade de Aveiro Departamento de Química
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Fosforilação Múltipla Da Histatina 1

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica do Dr. Francisco Amado, Professor Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha família pelo incansável apoio.

o júri

presidente

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palavras-chave Histatina 1, saliva, fosforilação, HPLC-ESI-MS, MALDI-TOF-MS.

resumo

As histatinas pertencem a uma família de peptídeos básicos ricos em histidinas que se encontram na saliva humana (Oppenheim et al., 1988). As principais histatinas, designadas histatinas 1 e 3, são codificadas pelos genes HTN1 e HTN2, localizados no cromossoma 4q13 (Sabatini et al., 1993). A histatina 3 é submetida a uma fragmentação pré-secretória, presumivelmente pela acção de uma convertase *furin-like*, e cria pelo menos 24 diferentes histatinas menores. Pelo contrário, a histatina 1, um peptídeo de 38 resíduos de amino ácidos, que comparte largamente a sua sequência com a histatina 3, não é submetida a fragmentação, provavelmente devido à ausência da sequência consenso da convertase (Castagnola et al., 2004). A histatina 3 e alguns dos seus fragmentos, em particular a histatina 5, têm propriedades antifungícas e antimicrobianas potentes, enquanto que o papel da histatina 1 ainda não foi bem estabelecido. Este estudo descreve os resultados obtidos na caracterização estrutural dos derivados poli-fosforilados da histatina 1 detectados por HPLC-ESI MS e MALDI-TOF-MS na saliva humana.

keywords Histatin 1, saliva, phosphorylation, HPLC-ESI-MS, MALDI-TOF-MS.

abstract Histatins belong to a family of basic histidine-rich peptides found in human saliva (Oppenheim et al., 1988). The major histatins, namely histatins 1 and 3, are codified by the genes HTN1 and HTN2, localized on chromosome 4q13 (Sabatini et al., 1993). Histatin 3 is submitted to a pre-secretory fragmentation, presumably by the action of a furin-like convertase, and it generates at least 24 different minor histatins. On the contrary, histatin 1, a peptide of 38 amino acid residues, largely sharing its sequence with histatin 3, is not submitted to fragmentation, probably due to the lack of the convertase consensus sequence Castagnola et al., 2004). Histatin 3 and some of its fragments, particularly histatin 5, show powerful antifungal and antimicrobial properties, whereas the role of histatin 1 has not been established. This study describes the results on the structural characterization of poly-phosphorylated derivatives of histatin 1 detected by HPLC-ESI MS and MALDI-TOF-MS in human saliva.

"Everything that a scientist does is a function of what others have done before him; the past is embodied in every new conception and even in the possibility of it being conceived at all."

Sir Peter Medawar (1979)

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INTRODUCTION

1. Saliva

Saliva is defined as a fluid present in the oral cavity, produced in and secreted by three pairs of major salivary glands – parotid, submandibular and sublingual – and numerous minor glands. Salivary gland secretion is regulated through the activity of the sympathetic and parasympathetic nerves to the gland, and their neurotransmitters (Izutsu, 1989).

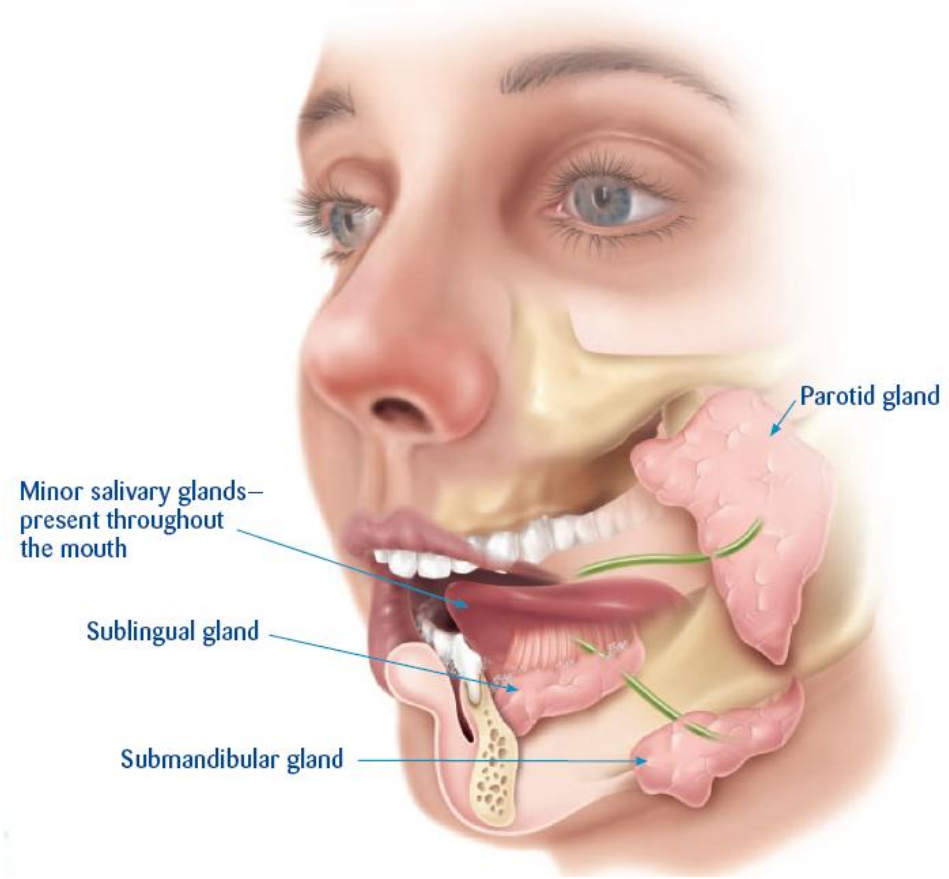

Figure 1 – Salivary glands (GlaxoSmithKline Consumer Healthcare).

Both communication and alimentation may be compromised when salivation is abnormal. The situation of having low salivary secretions (Sjogren's syndrome) reveals difficulty on speaking, chewing, forming a food bolus, and swallowing. In addition, there is a rapid and substantial increase in caries and mucosal infection, and taste and soft tissue complaints are also more frequent (Fox, 1989).

People who are naturally resistant to caries (regardless of fluorides and diet) have enhanced salivary protective mechanisms that include increased ability to produce base in plaque, a more effective means of bacterial aggregation and decreased pellicle permeability (Mandel, 1989).

Nevertheless saliva science has initiated its track relatively late in the history of medicine. Centuries before Irwin Mandel, a professor emeritus of Columbia University, grand saliva researcher and an historian on this matter, started his work in the laboratory, physicians thought the salivary glands were lowly modest excretory organs – ridding the body of toxins and evil spirits from the brain, and for that reason they would dose patients with poisonous dichloride of mercury, causing saliva to pour from the mouth. Even in the last century, scientists only got serious with saliva well after they'd tackled other body fluids such as blood. Like Mandel once said, "Saliva doesn't have the drama of blood, it doesn't have the integrity of sweat, it doesn't have the emotional appeal of tears" (Mestel, 2006). But from the 1950s onward, salivary research has been marked by a series of changing perceptions as new techniques and technologies have illuminated the complexities of the secretory mechanism, salivary composition, and function. Saliva rapidly broadened from the idea of being simply a digestive

fluid composed of salts, amylase, and mucin to cover a wide spectrum of protective proteins with the dual responsibility of protecting both hard and soft tissues.

As early as 1965, the use of several collecting devices to harvest the secretions from individual glands and the new separation techniques brought to very complex patterns (Mandel *et al.*, 1965) and it became clear that the major amino acid in saliva was proline and, more distinctively, that proline, glycine, and glutamic acid in a ratio of 3:2:2 was pervasive in both parotid and submandibular secretions as well as in the separated anionic and cationic fractions. In time, the basic proline-rich glycoprotein of parotid saliva was characterized (Levine *et al.*, 1969) and then came full recognition of the proline-rich family of proteins, which dominate saliva (Oppenheim *et al.*, 1971).

The genetic polymorphism of these proteins was quickly recognized (Azen and Oppenheim, 1973). In turn, we were set up to the tyrosine-rich protein, statherin (Hay, 1973), the cysteine-rich (Shomers *et al.*, 1982a), and the histidine-rich (MacKay *et al.*, 1984; Oppenheim *et al.*, 1986).

In the 1950s and early 1960s described an acquired, essentially bacterial-free, cuticle or pellicle on enamel that was distinct from the developmental cuticle (Nasmyth's membrane). During the 1960s, in vitro studies were begun on the adsorption of whole saliva to various forms of hydroxyapatite, including ground enamel (Hay, 1967). Selectivity of specific proteins, mainly anonic glycoproteins and phosphoproteins, was repeatedly noted. The acquired pellicle of selectively adsorbed proteins with a high affinity for hydroxyapatite coupled with neutral

lipids, glycolipids, and phospholipids forms a primitive membrane that provides a diffusion barrier that limits acid penetration and mineral exit as well as providing a lubricating film against excessive wear. Saliva in its insoluble form is as important to the maintenance of tissue integrity as is the parent fluid itself.

One of the greatest saliva stories of the 1960s was also the identification and characterization of secretory IgA in the secretions. With more specific antisera, Tomasi and Zigelbaum (1963) showed that IgA, not IgG, predominated, and then Tomasi *et al.* (1965) showed that there was a distinct immune system common to all external secretions bathing mucous membrane surfaces.

During the 1970s, not only were many salivary proteins identified and quantitated, but the structure and function of several of the protein families was elucidated - the isoamylases (Mayo and Carlson, 1974), statherin (Schlesinger and Hay, 1977), and the proline-rich proteins (PRPs) (Oppenheim *et al.*, 1971; Bennick, 1977). In the 1980s, the cysteine-rich proteins (Shomers *et al.*, 1982a-c) and the histidine-rich proteins (Oppenheim *et al.*, 1986, 1988), cystatins and histatins, namely, were characterized.

The digestive functions were seen to be secondary, rather than having a primary importance, mainly for preparing the food bolus for mastication, for swallowing, and for normal taste perception (Mandel, 1987). It became increasingly apparent that the multivariate mix of salivary constituents provides an effective set of systems for lubricating and protecting the soft and hard tissues.

Salivary mucins with their low solubility, high viscosity, elasticity, and adhesiveness are highly effective in helping to maintain mucous membrane integrity, acting to protect the soft tissues against dessication, penetration, ulceration, and potential carcinogens. Other salivary proteins can aid in this protective capacity; examples are PRPs that bind tannins, etiologic agents in esophageal cancer, cystatins, which are effective inhibitors of cysteine-proteases, and antileukoprotease, an inhibitor of elastase and cathepsin (Mandel, 1987, 1989).

The combination of the multiple host defense functions in saliva shows a protective repertoire that goes beyond antibacterial activity to include antifungal, antichlamydial, and antiviral properties as well (Mandel, 1993).

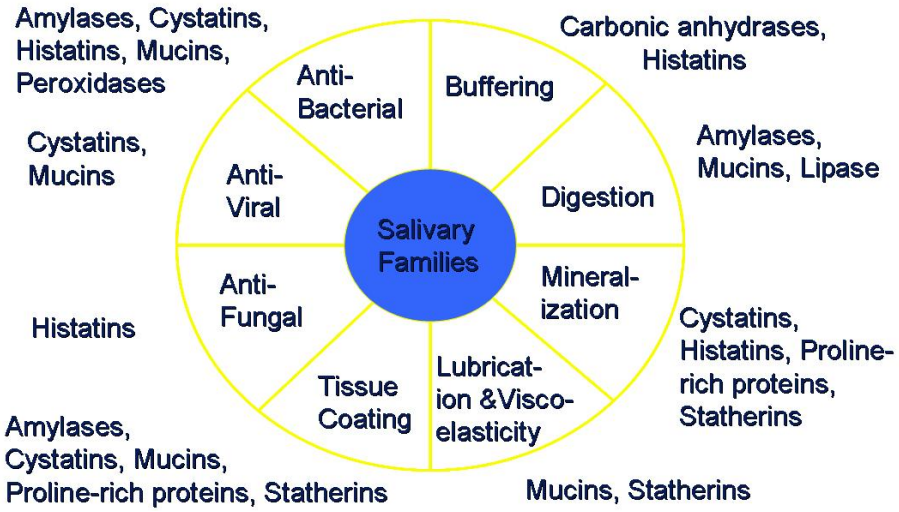

Figure 2 – Multifunctionality of salivary families (adapted from Levine, 1993).

2. Salivary Peptides

Based on several works, the salivary peptides are usually classified in five different main classes according to their intrinsic properties: proline-rich peptides (PRP), cystatins, defensins, statherin and histatins (Van Nieuw, 2004); figure 3.

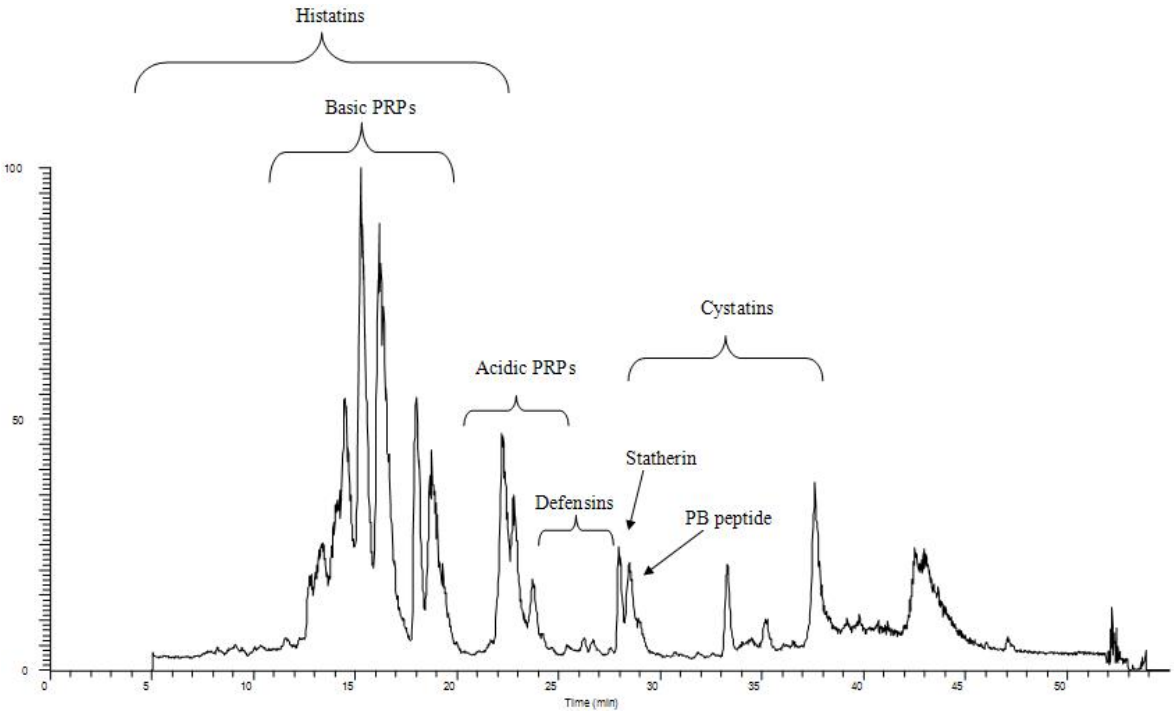

Figure 3 – RP-HPLC-MS profile of the acidic fraction from human saliva.

2.1. Proline-Rich proteins (PRPs)

PRPs, designation due to their high content of proline residues, are characterized in three different forms: acidic, basic and glycosilated (Hardt *et al.*, 2005).

2.2. Cystatins

Cystatins cover five major isoforms: S, C, D, SA and SN. They are powerful inhibitors of cysteine peptidases such as cathepsins B, C, H and L, and have strong bactericidal and virucidal properties.

2.3. Defensins

The defensin family is found in plasma, wound fluid, intestine and skin of human. Its antibiotic, antifungal and antiviral properties are known (Amado *et al.*, 2005).

2.4. Statherin

Statherin is a multifunctional molecule with high affinity for calcium phosphate minerals such as hydroxyapatite that contributes to maintain the appropriate mineral solution dynamics of enamel. Jensen *et al.* (1991) characterized three isoforms of statherin: lacking one phenylalanine residue at the C-terminus (SV1), lacking residues 6-15 (SV2), and SV2 lacking phenylalanine residue at the C-terminus (SV3). Vitorino *et al.* (2004) proposed an additional statherin isoform corresponding to the loss of one aspartic acid residue at the N-terminus.

2.5. Histidine-Rich Peptides

The parotid and submandibular glands of humans secrete a family of small, mostly cationic, histidine-rich proteins called histatins (Oppenheim *et al.*, 1988). The current preferred peptide chemical information and nomenclature for histatins was proposed by Troxler *et al.* (1990).

Histatins show strong antifungal activities and appear to actively participate in teeth remineralization processes (Edgerton, 2000). They have been also shown to be tannin-binding proteins in human saliva (Yan and Bennick, 1995). Histatins 1, 3, and 5 comprise 85 to 90% of the total histatin proteins and are termed the major histatins. All 3 major histatins have candidicidal and bactericidal activity.

There are two human genes recognized as responsible for the synthesis of histatin 1 and histatin 3, HTN1 (HIS1) and HTN2 (HIS2), respectively, localized on the chromosome 4, band q13 (Sabatini *et al.*, 1989); Figure 4.

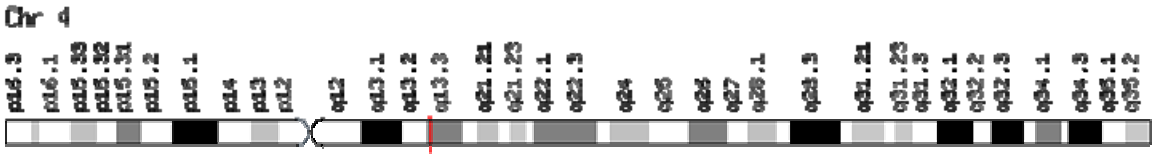

Figure 4 – Localization of the genes HTN1 and HTN2. Chromosome 4, band q13 assigned in red.

Histatin 1 is a phosphoprotein of 38 amino acids, with the modification on Ser-2, whereas histatin 3 has 32 amino acids with a very similar sequence to histatin 1, although not phosphorylated (Oppenheim *et al.*, 1988).

Troxler *et al.* (1990) identified in human saliva histatin 2, a peptide corresponding to the carboxyl terminal 26 residues of histatin 1, and nine peptides, all related to the sequence of histatin 3, named histatins 4-12. Except histatin 2, the other minor histatins are likely originated by proteolytic cleavages from histatin 3. Amongst there's histatin 5, a major fragment present in human saliva at high concentration, showing a sequence identical to the first 24 amino acids of histatin 3. This peptide is the most potent member with respect to its fungicidal activity against the oral opportunistic pathogen *Candida albicans* species, with respect to all the other histatins (Xu *et al.*, 1991).

The study of Castagnola *et al.* (2004) allowed the detection of new histatin peptides: 24 peptides including, with the exception of histatin 4, all the known histatin 3 fragments, namely histatins 5-12, and the new peptides corresponding to 1-11, 1-12, 1-13, 5-13, 6-11, 6-13, 7-11, 7-12, 7-13, 14-24, 14-25 15-25, 26-32, 28-32, 29-32 residues of histatin-3 (Figure 5). All these fragments showed a complex proteolytic pathway involving histatin 3. The absence of proteolytic activity on histatin 1 could be attributed to the lack of an amino acid equivalent to the Arg-25 residue of histatin 3, which appears to represent the first crucial cleavage site for this peptide.

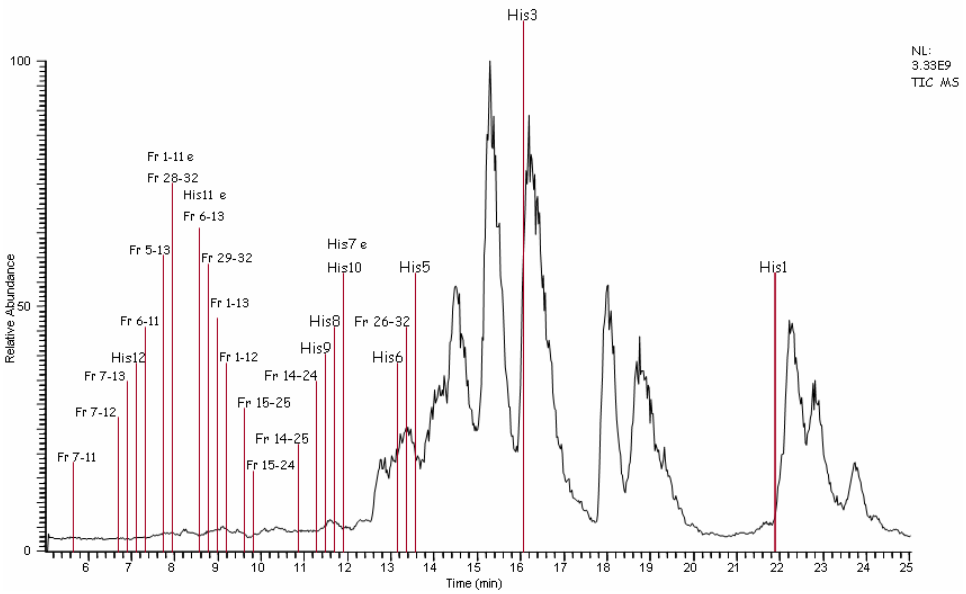

Figure 5 – Time range enlargement (5–25 minutes) showing the approximate elution position of all the histatins and histatin 3 fragments detected by Castagnola *et al.* (2004).

3. Histatin 1

Although the role of histatin 3 and some of its fragments, particularly histatin 5, is known as being antifungal and antimicrobial, the function of histatin 1 has not been well established

HTN1, the gene that synthesizes histatin 1, is expressed at very high level and is defined by 1866 cDNA clones. The gene contains 31 introns (AceView, 2006).

The sequence of histatin 1 (Figure 6) is largely shared by the sequence of histatin 3, but it is not submitted to fragmentation, as previously referred (Castagnola *et al.*, 2004).

DSHEKRHHGY RRKFHEKHHS HREFPFYGDY GSNYLYDN

Figure 6 – Sequence of amino acids residues of histatin 1. Underlined serine (S) stands for the phosphorylated residue.

Recently, Onnis *et al.* (2005) presented in the Symposium of the Italian Biochemistry Society at Cagliari the results of HPLC-MS-ESI spectra of an acidic solution of whole saliva unveiling the presence of a combination of masses (Table 1) consistent with the multiple phosphorylation of histatin 1.

Peptide	Experimental average mass	Theoretical average mass
histatin 1 non-phosphorylated	4848 ± 0.8	4848.2
histatin 1 mono-phosphorylated	4928 ± 0.8	4928.2
histatin 1 di-phosphorylated	5008 ± 0.8	5008.2
histatin 1 tri-phosphorylated	5088 ± 0.8	5088.2
histatin 1 tetra-phosphorylated	5168 ± 0.8	5168.2
histatin 1 penta-phosphorylated	5248 ± 0.8	5248.2

Table 1 – Multiple phosphorylation of histatin 1 determined by HPLC-MS-ESI (Onnis *et al.* 2005).

The presence of the multiple phosphorylations on the molecule may be a clear indication of the performed function. Therefore, the main purpose of this work is to establish the consistency of the histatin 1 multiple phosphorylation, and, if possible, determine the sites of phosphorylation occurrence.

3.1. Phosphorylation identification strategy

Protein phosphorylation is a common post-translational modification in both prokaryotes and eukaryots. The cellular processes in which it's involved includes cell signaling, growth and metabolism.

The phosphorylation of proteins is catalyzed by kinases (phosphorylation) and phosphatases (dephosphorylation). In eukaryotes, this modification occurs on serine (~90%), threonine (~10%) and tyrosine residues (~0.05%). Although the

tyrosine phosphorylation is less common, reversible phosphorylation on this residue plays an important role in signal transduction cascades.

There are available several methods for phosphorylation detection and characterization. For instances, metabolic labeling of phosphorylated proteins with [^{32}P] phosphate is a method by excellence for detection. However, subsequent sequence data must be acquired via Edman degradation or mass spectrometry.

Major achievements have been accomplished in the field of mass spectrometry, namely the mild ionization techniques of MALDI and ESI, which have been honoured in 2002 by the Nobel Prize in chemistry. For the task of protein identification, nowadays MALDI and ESI ionization sources are commonly coupled to quadrupol, time-of-flight or ion trap for ion characterization.

Usually, the proteins are cleaved with chemicals or proteases to obtain smaller peptide fragments. Trypsinization is the most common strategy used. The layout of ESI instruments is highly suitable for molecule fragmentation by collision with an inert gas, which can be used to determine the amino acid sequence of the peptide from the mass differences of the fragments. Alternative cleavage conditions, such as the use of other proteases like proteinase K have been described (Wu *et al.*, 2003) and proven superior to trypsin in practice.

One mass spectrometric method for phosphopeptide analysis involves detecting the neutral loss of H_3PO_4 (-98 Da) or HPO_3 (-80 Da) from the phosphorylated residue (DeGnore and Qin, 1998) by tandem mass spectrometry. Another method involves precursor scanning for specific marker ions, such as

m/z 63 (PO_2^-) and 79 (PO_3^-), in the negative mode, however it makes sequencing more difficult; otherwise, there's the possibility of using skimmer collision-induced dissociation (CID) (Zolodz and Wood, 2003).

Taking the mass range of common instruments, and the MS/MS and PSD capability of ESI and MALDI, respectively, into account, analysis of the probable several phosphorylated histatin 1 digests with trypsin, proteinase K and protease V8 was carried out to identify the different phosphorylations on histatin 1 by detecting the typical neutral losses.

4. Research goals

The goals for this research were to identify histatin 1 multiple phosphorylation, and relative determination of phosphorylation site using mass spectrometry.

Salivary histatins were extracted from whole saliva, followed by their separation and identification by HPLC and HPLC-MS. Due to the complexity of the peak pattern, each histatin 1 was not completely purified, instead there was a colletion of five pools with different composition of the poly-phosphorylated peptides. After the HPLC separation, each pool undergoes proteolytic digestion and mass peptide mapping to identify and determine the post-translational modification. To confirm these assignments, MS/MS and PSD were performed on the different pools with the poly-phosphorylated histatins 1 and its proteolytic digest fragments.

METHODS

1. Reagents

All common chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba, (Milan, Italy), Merck (Damstadt, Germany) and Sigma Aldrich (St. Louis, MI, USA). Chromatographic eluents were from Carlo Erba.

The benzoylated dialysis cellulose membrane with molecular weight cut off of 2000 Da was purchased from Sigma Aldrich. Immobilized trypsin and protease V8 were obtained from Pierce Biotechnology (Rockford, IL, USA), and proteinase K was acquired from Promega (Madison, WI, USA). CHCA matrix and MALDI peptide calibration standards were purchased from Bruker Daltonics (Bremen, Germany).

2. Apparatus

The HPLC apparatus was a ThermoFinnigan Surveyor Plus (San Jose, USA). The HPLC-ESI-IT-MS apparatus was a ThermoFinnigan Surveyor HPLC connected by a T splitter to a PDA diode-array detector and to Xcalibur LCQ Deca XP Plus mass spectrometer. The mass spectrometer was equipped with an electrospray ion (ESI) source.

The chromatographic columns were a Vydac (Hesperia, CA, USA) C_8 column with 5 μm particle diameter (dimensions 150x2.1 mm) and an Agilent Hypersil BDS-C_{18} column with 3 μm particle diameter (dimensions 100x4.0 mm). MALDI-TOF-MS was performed using the Autoflex Bruker Daltonics apparatus (Bremen, Germany).

3. Methods

3.1. Sample collection and treatment

Every volunteer had its saliva analyzed and characterized, in order to establish it as a standard saliva profile (absent of abnormal peptides). A massive preparation of human saliva (500 mL) was done after. The whole human saliva was collected in three different days between 10a.m. and 14p.m from seven normal adult informed volunteers according to a standard protocol (Castagnola *et al.*, 2001). An acidic solution (0.2% TFA) was immediately added to salivary samples in an ice bath at a 1:1 v/v ratio and the solution centrifuged at 8000 g for 5 min. After centrifugation the supernatant was separated from the precipitate.

3.2. Histatins Isolation Using Zinc Precipitation

Zinc chloride was added from a solution 5 mM to the supernantant obtained from the treated saliva with the acidic solution to yield a final concentration of 500 μM. The pH was subsequently raised to 9.0 with a solution 3 M NaOH and the sample incubated at 4ºC for 20 minutes. After centrifugation at 16,000g for 20 minutes, the supernatant was removed and stored at 4ºC. The pellet containing different precipitated peptide–metal complexes was washed once in the zinc chloride solution.

Washed pellet was dispersed in distilled water, dissolved by acidifying the solution with 3 M HCl and then subjected to dialysis in a benzoylated dialysis cellulose membrane with molecular weight cut off of 2000 Da using the buffer acetate 30 mM / EDTA 10 mM, pH 5.6.

The dialysis had two days duration and the buffer was changed four times. Supernatant was then removed from inside the membrane and lyophilized.

3.3. HPLC Purification

The histatins complex in the lyophilized sample obtained through the protocol described in section 3.2 was partially purified by chromatographic separation.

The following solutions were utilized for the reversed-phase chromatography: (eluent A) 0.056% aqueous TFA and (eluent B) 0.05% TFA in acetonitrile-water 80/20 (v/v). The gradient applied arrived to 10% of B in 1 minute and the was linear from to 37% B in 36 minutes, at a flow rate of 0.90 mL/min.

The lyophilized samples were dissolved in 0.2% TFA. Several HPLC analyses were done by injecting each time 200 μL. The column eluate was collected in fractions. Those containing the various histatins were evaporated to dryness in the lyophilizer, taken up in 0.2% TFA, and re-chromatographed in the HPLC-MS in order to establish the histatins composition in each different fraction by verification of the mass pattern.

3.4. Enzymatic Digestion

The different compositional histatins fractions collected by RP-HPLC were submitted to several digestions and then analyzed by RP-HPLC-ESI and MALDI-TOF MS.

3.4.1. Trypsin

Tryptic digestion was carried out according to the supplier's instructions (Pierce Biotechnology). Briefly, the lyophilized powders from each of the pools collected (~30-80 μg) were dissolved into 250 μL of the digestion buffer (ammonium bicarbonate 0.1 M, pH 8.0) and incubated at 37°C for 3 h with 50 μL of immobilized trypsin. At the end of digestion, immobilized trypsin was separated by centrifugation at 3000g. Each solution was lyophilized, dissolved in 0.2% TFA and submitted to RP-HPLC-ESI MS and MALDI-TOF-MS analyses.

3.4.2. Proteinase K

The digestion with proteinase K was performed under the supplier's instructions (Roche Biochemicals). The lyophilized samples from each fraction pool (~30-80 μg) were dissolved into 260 μL of the digestion buffer (TRIS-HCl 50 mM, pH 8.0, 3 mM $CaCl_2$) and incubated at 56°C for 3 h with proteinase K at a final concentration of 50 μg/ mL. At the end of digestion, proteinase K was

inhibited by addition of PMSF (phenylmethylsulfonyl fluoride) at a final concentration of 5 mM. Each solution was then lyophilized, dissolved in 0.2% TFA and put forward to RPHPLC-ESI MS and MALDI-TOF-MS analyses.

3.4.3. Protease V8

The enzymatic digestion with protease V8 was executed following the supplier's instructions (Pierce Biotechnology). The samples from each of the collected fractions (~30-80 μg) were dissolved into 160 μL of the digestion buffer (buffer 3 from the suppler's kit; specificity for cleavage at the carboxy terminus sides of both glutamic and aspartic acid) and incubated at 37°C for 7 h with 40 μL of immobilized protease V8. At the end of digestion, immobilized enzyme was separated by centrifugation at 3000g. Each product solution was lyophilized, dissolved in 0.2% TFA and submitted to RP-HPLC-ESI MS and MALDI-TOF-MS analyses.

3.5. MS Experiments

Each of the different digested fraction pools was submitted to mass spectrometric experiments (ESI and MALDI), in order to find out the peptide fragments obtained in the enzymatic reactions with trypsin, proteinase K and protease V8. All spectra were obtained in the positive ion mode.

3.5.1. ESI-MS

The following solutions were utilized for the reversed-phase chromatography: (eluent A) 0.056% aqueous TFA and (eluent B) 0.05% TFA in acetonitrile-water 80/20 (v/v). The gradient applied was linear from 0 to 55% in 40 min, at a flow rate of 0.300 mL/min. The T splitter addressed about a flow-rate of 0.20 ml/min towards the diode array detector and a flow-rate of 0.10 ml/min towards the ESI source. The diode array detector was usually settled at a wavelength of 214-276 nm. Mass spectra were collected every 3 millisecond in the positive ion mode. MS spray voltage was 4.50 KV and the capillary temperature was 220°C.

3.5.2. MALDI-MS

The lyophilized digestion products were purified using ZipTip C18 micropipet tips following the protocol for desalting/purification provided by the manufacturer. The samples dissolved in 0.1% aqueous TFA solution were mixed with the CHCA (α-cyano-4-hydroxycinamic acid) matrix solution and deposited onto the stainless steel target of the MALDI instrument, according to the dried droplet method (Nordhoff *et al.*, 2003). To prepare matrix solutions, saturating amount of CHCA was added to acetonitrile:water (33:67, v/v) containing 0.1% TFA. Calibration was performed using peptide standard mixture (angiotensin I and II, substance P and bombesin). MALDI-TOF mass spectra were acquired with a pulsed nitrogen laser (337 nm) in positive, either linear or reflector, mode. In linear mode an ion source of 20 kV, a pulsed ion extraction time of 350 ns, a detector gain voltage of 1300 V and a laser frequency of 5 Hz were used. In positive reflector mode, spectra were obtained with an ion source of 19 kV, a pulsed ion extraction time of 150 ns, a detector gain voltage of 1400 V and a laser frequency of 5 Hz. About 400 scans were averaged for each spectrum to improve signal-to-noise ratio.

3.6. Tandem-MS Experiments

Tandem mass spectrometric experiments were done to any ions suspected of having the interest modifications to find and verify structural information relating to the sequence (and modification itself) of the fragments obtained from the digests.

3.6.1. ESI-MS/MS

Tandem-MS experiments were performed by detection of parent ions with a peak width of 2-4 m/z values with 40-60% of the maximum activation amplitude.

3.6.2. MALDI-PSD

PSD experiments were realized in the positive-ion mode using the segmented approach, where the voltage of the ion reflector is stepped down gradually (Spengler, 1991).

After MALDI-MS analysis was done, a peak was time selected and analyzed by PSD. The instrument was first calibrated in the normal MALDI mode and then in the PSD mode. In addition, the ion selector window must be calibrated to select the specific ion of interest for PSD.

The PSD acquisition was composed of 19 individual segments that were later stitched together by the FAST algorithm. The reflector voltage was varied from 19.00 kV to 0.56 kV. The ion ejection voltages at IS1 and IS2 were set to 18.00 kV and 15.75 kV, respectively, and the lens were set to 7.40 kV. The latter three voltages were kept constant throughout the experiment with only the reflector voltage being changed for each segment.

3.7. Data analysis

Deconvolution of average ESI mass spectra was automatically performed by MagTran 1.0 software (Zhang and Marshall, 1998). Flex control software was used for data acquisition and processing of MALDI mass spectra. Experimental mass values were compared with average theoretical mass values using the PeptideMass program available at the Swiss-Prot (http://us.expasy.org/tools) data bank (histatin 1 code: P15515). Mass values determined on the different digested samples were compared with the expected average and monoisotopic values calculated using the PeptideMass program. Phosphorylation sites were deduced by NetPhos program, available at the Center for Biological Sequence Analysis (Blom *et al.*, 1999) (http://www.cbs.dtu/dk/services/NetPhos/). Generation of theoric MS/MS spectra were performed utilizing the MS-Product program, available at the Protein Prospector site (http://prospector.ucsf.edu/).

RESULTS AND DISCUSSION

1. Sample collection and treatment

The salivary sample pre-treatment with acidic solution caused the precipitation of amylases, α-mucins, and other high molecular mass salivary proteins. Furthermore, the treatment reduced at a minimum the action of endogenous and exogenous salivary proteases and, consequently, the artifact occurrence.

All the histatins were soluble in the acidic solution, as it was confirmed by an RP-HPLC analysis (Figure 7).

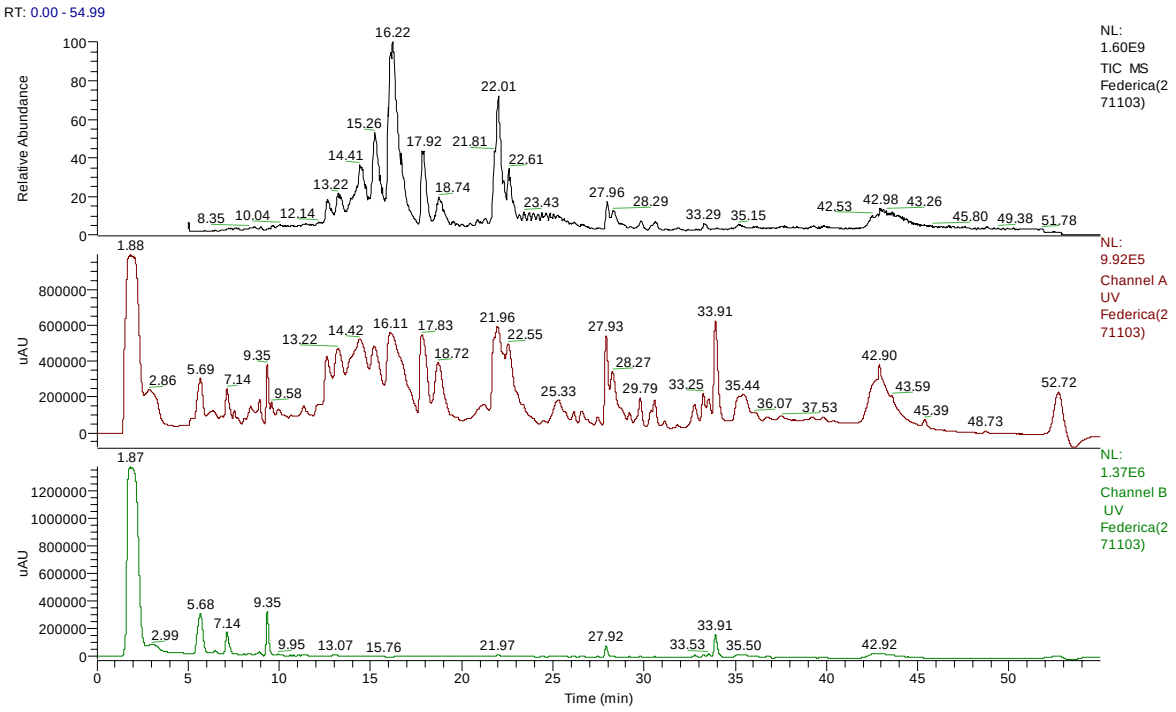

Figure 7 -HPLC-MS spectrum of whole saliva acidic fraction. Upper panel states for the total ion current recorded, and the two bottom panels show the UV profile at 214 and 276 nm.

In the upper panel the profile of total ion current (TIC) recorded by the mass spectrometer is reported. During the HPLC separation also the UV profiles at 214 and 276 nm were collected. During the first three-five minutes of elution the eluent was not addressed towards the ESI-MS apparatus in order to avoid damages of the ion-trap mass spectrometer, due to the elevated concentration of electrolytes.

In the aim to isolate those pertaining histatin fragments, it was adopted the strategy described in the section 3.2 (Oppenheim et al., 1989).

2. Histatins Isolation Using Zinc Precipitation

When zinc chloride is added to a final concentration of 500 μM to the treated saliva collected and pH raised to 9.0, a white precipitate is formed due to the creation of insoluble metal complexes. It contains most of the low molecular weight proteins, while the higher molecular weight proteins remain in the supernatant.

Oppenheim et al. showed (2001) that this precipitation is zinc dependent and not due to a salt effect, although approximately 10% of the histatins remains in the supernatant. The complexed histatins are mainly 1, 3, 5 and 6. This metal binding property is accredited to the high quantity of histidine, serine, tyrosine and aspartic and glutamic acids known to participate in the metal ions coordination. Also statherin and PRPs precipitate.

Since PRPs co-elute with some histatins, it was performed a dialysis of the acidified solution obtained from the metal complexes precipitate. A new precipitate was formed containing the almost all of the undesired PRPs (HPLC-MS analysis of the dialysis supernatant showed the presence of PRPs, such as Pb peptide and PRP glycosilated derivatives).

3. HPLC Purification

During HPLC purification it was detected a peak corresponding to histatin 1 that also includes the poly-phosphorylated derivatives (non-, mono-, di-, tri-, tetra-, and penta-phosphorylated), as revealed by SIM (selected ion monitoring) strategy. It's also perceptible their low quantity (Figure 8).

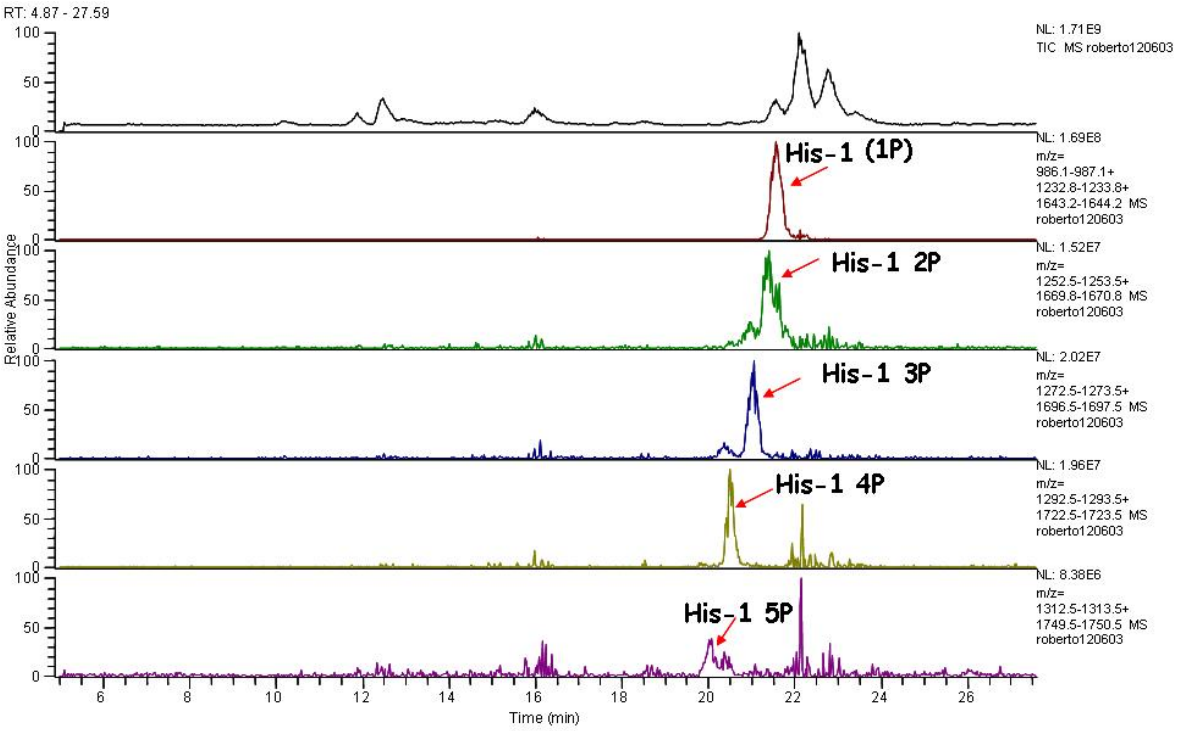

Figure 8 – Chromatographic profile of the different poly-phosphorylated histatins 1 by means of SIM strategy.

In fact, the HPLC purification of the six different poly-phosphorylated histatins 1 cannot be fully completed.

After different tests, the gradient was chosen, in order to reach a satisfactory compromise between peak resolution and analysis time. It was obtained, instead of a single peak, a not resolved complex peak that allowed the division of it into different pools.

A typical separation obtained on histatins sample is reported in figure 9.

The number of theoretical plates per meter (*N*/m) on histatin 1 peak was maintained trough all the chromatographic analysis, with no changing of the guards column, showing that the column performance didn't decline with the used conditions.

Histatin 1 contains in its sequence five tyrosines, being tryptophan absent, whereas proline-rich proteins lacking in these aromatic amino acids. Tyrosine and tryptophan are the only two amino acids responsible for a sensible protein absorbance over 260 nm. Also disulfide bridges absorb at this wavelenght. The maximum of tyrosine absorbance is at 272 nm, whereas that of tryptophan is 280 nm, with a molar extinction coefficient that, under the HPLC conditions used, is approximately four-times higher than that of tyrosine.

The PRPs peaks were obviously detectable only at the non-specific 214 nm wavelength. Histatins peptides (in particular, the different polyphosphorylated histatins 1) were identified on the basis of polarity criteria and by following absorbance at 276nm.

Since histatin 1 chromatographic peak has complex pattern, it was divided into five different pools, which were lyophilized and submitted to HPLC-ESI-MS analysis, confirming the molecular mass of these peptides, within the experimental error characteristic of this analysis; the poly-phosphorylated histatins 1 found in each pool are reported in Table 2 (and figure 9). Analysis on the relative abundance showed that histatin 2 (mono-phosphorylated histatin 1) was about 10% with respect to histatin 1 and the poly-phosphorylated isoforms were all less than 2-3% in normal subjects. The tri- and tetra-phosphorylated isoforms were usually the most abundant, whereas the di-phosphorylated and the penta-phosphorylated isoforms were often under the detection limit of the method.

Table 2 – Human salivary poly-phosphorylated histatins 1 detected in the selected five pools.

HPLC Pool	Peptides
I	non-, mono-, di-, tri-, tetra- and penta-phosphorylated
II	non-, mono-, di-, tri-, tetra- and penta-phosphorylated
III	non-, mono-, di- and tri-phosphorylated
IV	non-, mono- and di-phosphorylated
V	non- and mono- phosphorylated

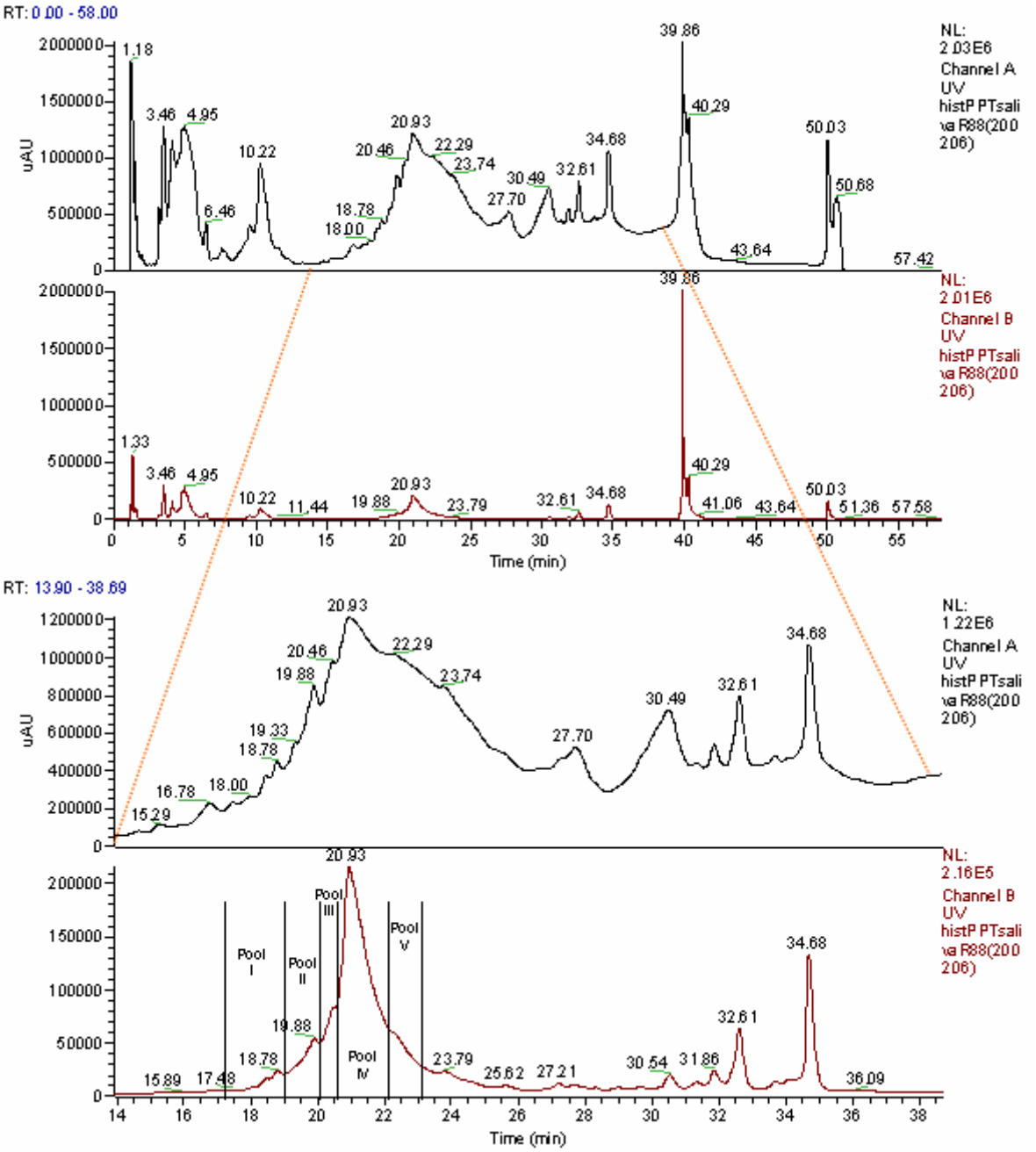

Figure 9 – Enlargement of the 14.0–39.0 min elution range showing the UV profile (214 and 276nm) of the HPLC chromatogram. The pools collected are shown.

4. Enzymatic Digestion

To confirm the hypothesis of the multiple phosphorylation of histatin 1, all pools with partially purified histatins were digested with trypsin, proteinase K and protease V8. The resulting digests were then submitted to HPLC-ESI-MS and MALDI-TOF analysis.

The theoretical digest masses were calculated considering all the cutting possibilities with each enzyme inside the known sequence of histatin 1. It was also taking in regard the possible multiple phosphorylations on the residues tyrosine and serine in a maximum of five phosphate groups in the fragment sequence.

The theoretical fragments were 97 for trypsin, 338 for proteinase K and 111 for protease V8 digestions. For each of the fragments it was created a layout for application in the Xcalibur program in order to facilitate the fragment search on the spectra of the digests.

5. MS and MS/MS experiments

The efficiency of ionization of phosphopeptides in peptide mixtures is a large problem in ESI-MS-based phosphopeptide screening. This inefficiency is probably due to competition between peptides, because most of them in an enzymatic digest of a phosphoprotein are not phosphopeptides, hence, even when they are phosphorylated at a high stoichiometry , they are only minor components of the overall mixture and have to compete with all the non-phosphorylated peptides. Additionally, although having the same sequence, the non-phosphorylated and the phosphorylated forms of a given peptide have different ionization efficiencies; since the phosphorylated form is more acidic, it is less efficiently protonated and as a result less efficiently ionized. Another issue of great importance is that cleavage is mainly directed to the weakest linkages, resulting on the neutral loss of the phosphate groups instead of the cutting on the peptide linkage, making identification and phosphorylation site location very challenging.

The peptide fragments were eluted from the column between 5-35 min and detected at 214 and 276 nm. After spectra analysis, measured peptide fragment masses were compared against theoretical digest masses.

Only those peptides having mass errors within the 90% confidence limit were considered as valid matches. Mass shifts related to 98 Da were considered as phosphorylation on serine residues. Mass shifts of 80 Da were considered as phosphorylation on tyrosine residues (DeGnore and Qin, 1998; Steen et al., 2006).

In this study, only peaks with a relative abundance and a signal-to-noise ratio greater than 3 were chosen for data analysis. All *m/z* data and fragment assignments for each peptide detected are provided in the Appendix.

From the 87 fragments found, 36 were phosphorylated.

In view of the complexity of protein digests, a tandem MS step is generally required for reliable phosphopeptide identification and is necessary for pinpointing of the phosphorylation site(s). The MS/MS experiments performed were 57.

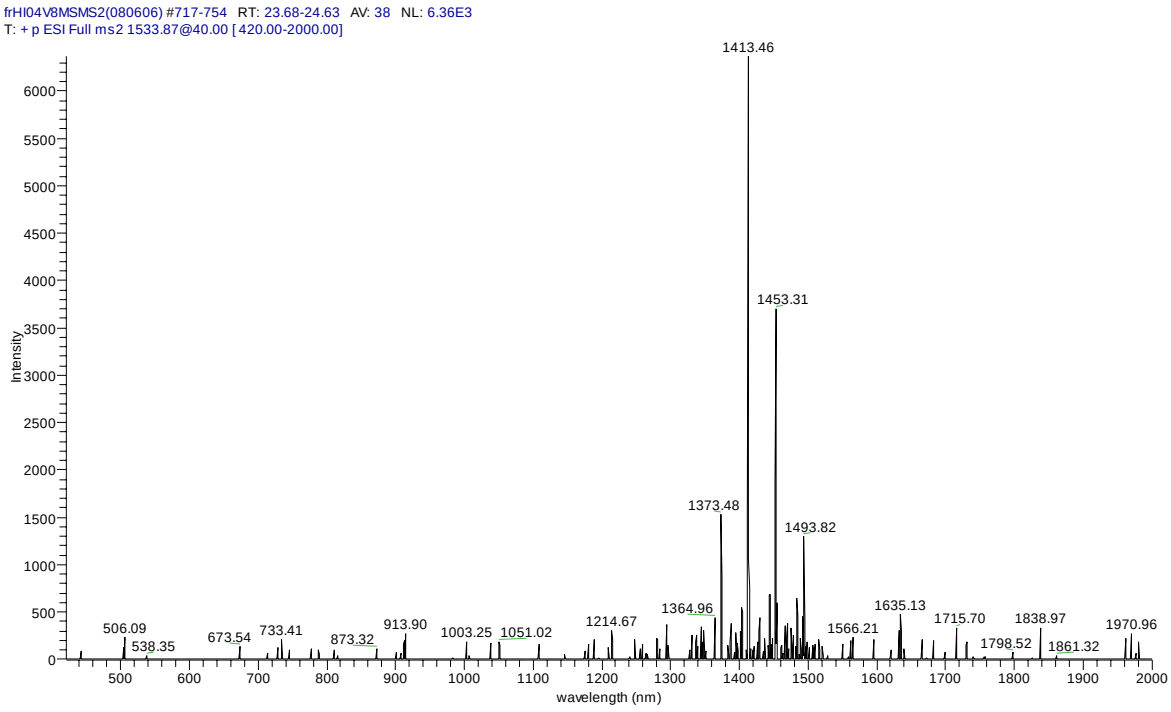

Figure 10 – MS/MS experiment for *m/z* 1533.87 (sequence K17-N38).

LC/MS/MS analysis of the digests located four phosphorylation sites. For example, within a fragment of the C-terminal region of histatin 1 (sequence K17-N38), being all of the phosphorylations located on the last four tyrosines of the sequence: 27, 30, 34 and 36,according with the fact that in the MS/MS spectra of the $[M+2H^+]^{+2}$ ion (*m/z* 1533.87) are visible four neutral losses of 40 Da (80 Da/2; typical loss of HPO_3 from a tyrosine residue), $[M-H_3PO_4+2H^+]^{+2}$, $[M-2H_3PO_4+2H^+]^{+2}$, $[M-3H_3PO_4+2H^+]^{+2}$, and $[M-4H_3PO_4+2H^+]^{+2}$, consistent with four phosphate groups in the tyrosine residues. Since the fragment considered has only four tyrosines in possibility, the sites identification is unequivocally (Figure 10). The fifth position of the phosphate group would be on Ser-2, as already established. The MS/MS experiment of the fragment m/z 829.4 Da (sequence D1-R12) support this fact, showing one loss of 98 Da (serine residues usually lose H_3PO_4) consistent with the reported phosphorylation on serine (Figure 11).

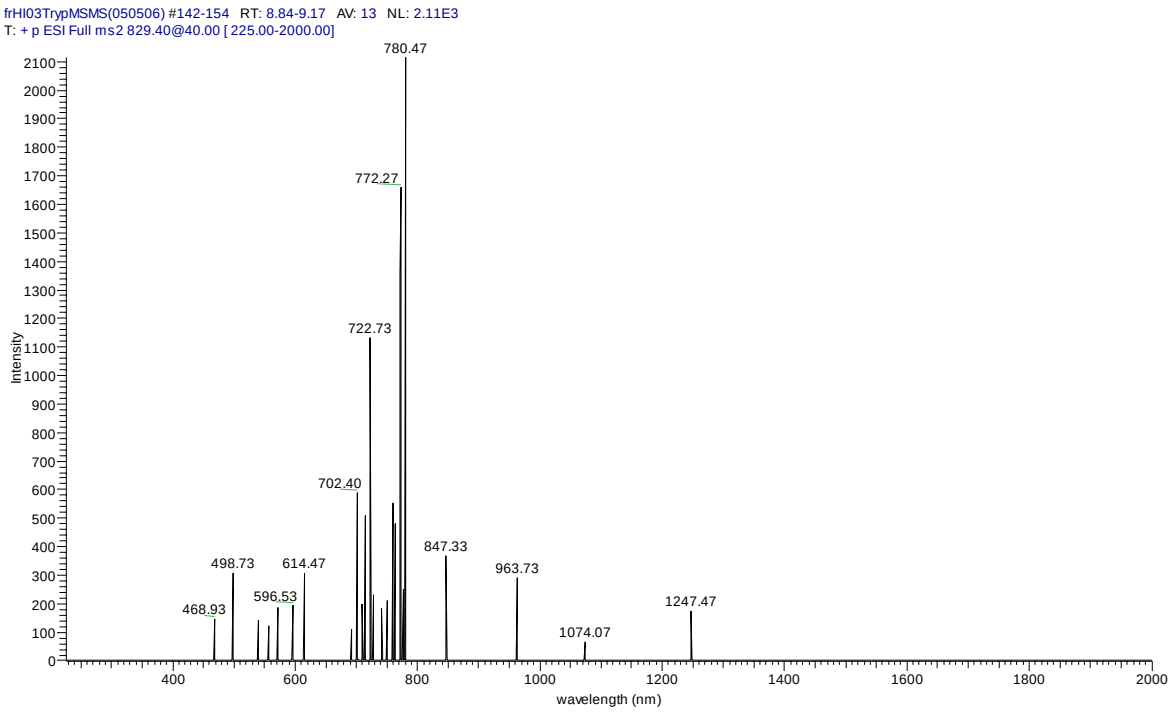

Figure 11 – MS/MS experiment for *m/z* 829.4 (sequence D1-R12).

Also the PSD experiment sustains the presence of the phosphorylated serine on the position 2 (Figure 12).

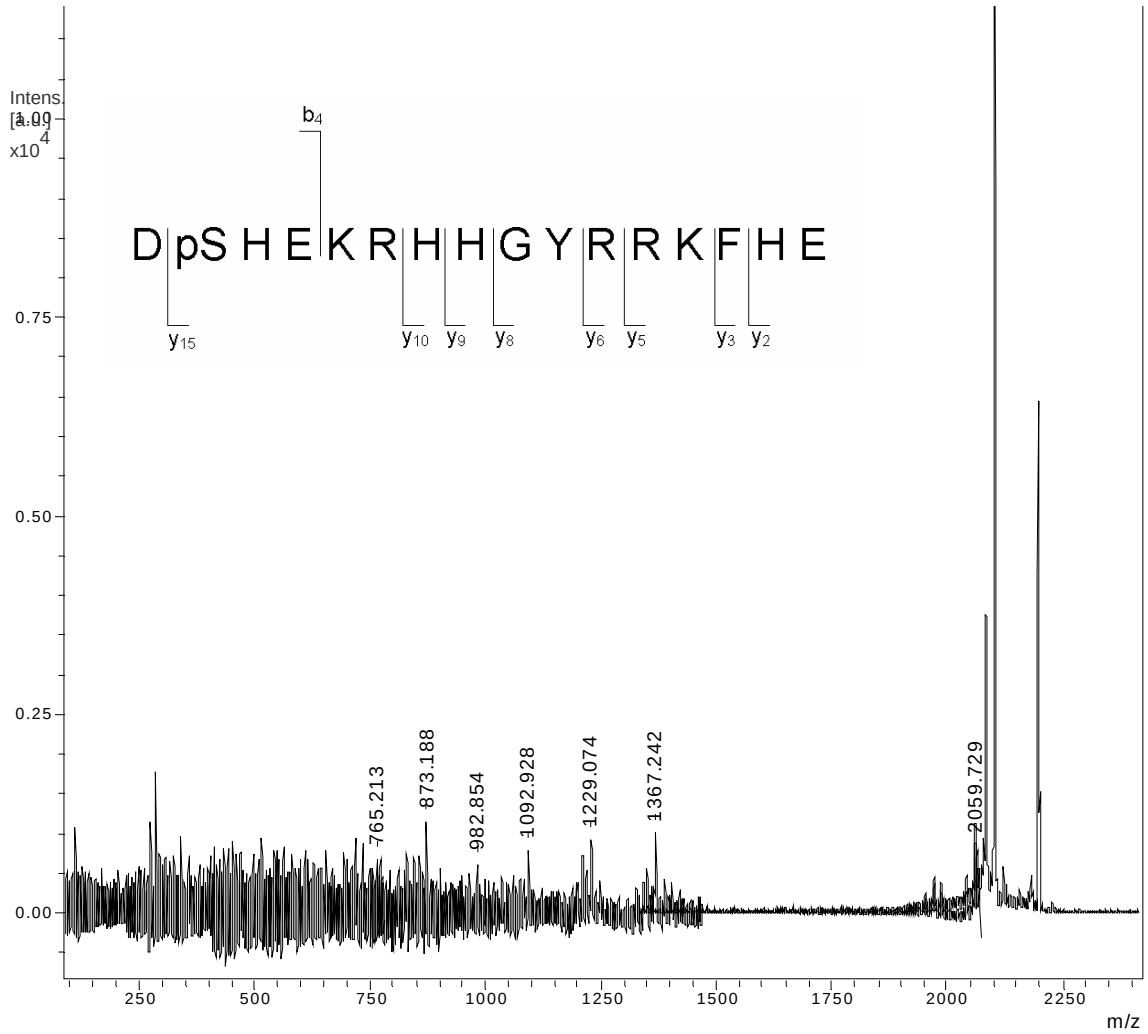

Figure 12 – PSD experiment on *m/z* 2199.14 (sequence D1–E16, phosphorylated on Ser–2).

The PSD analysis were not performed on the interest peptide fragments with the multiple phosphorylations on the last four tyrosines due to their low peak intensity and proximity to other fragment peaks. Instead, it was possible to carry it out for certain masses in agreement with phosphorylated fragments in the Ser-20 and 32 and Tyr-10. The spectra showed that the masses didn't correspond to the attributed fragment allowing to exclude the possibility of phosphorylation on those sites, also because all the digest fragments found ranging those residues were non-phosphorylated.

However, the position of these modifications for the di-, tri-, and tetra-phosphorylated peptides could not be unambiguously determined, given that each pool sample has a complex pattern and, therefore, cannot be said wich histatin 1 isoform originates some determined digested phosphorylated fragments.

6. Theoretical Phosphorylation Prediction

The NetPhos program allows the prediction of the potential phosphorylation sites, giving a high score for Ser-20 and lower scores for Ser-2 and Ser-32. However, all the derivatives of histatin 1, with the exception of histatin 2 (mono-phosphorylated histatin 1), are phosphorylated at Ser-2, probably by a Golgi (casein-like) kinase not included in NetPhos algorithm, which recognizes SX(E/Sphos) as principal consensus sequence (Brunati et al., 2000).

Taking into account the phosphorylation of tyrosine residues, the highest scores were obtained for 27, 30 and 36 positions and the lowest for 10 and 34 positions (Figure 13).

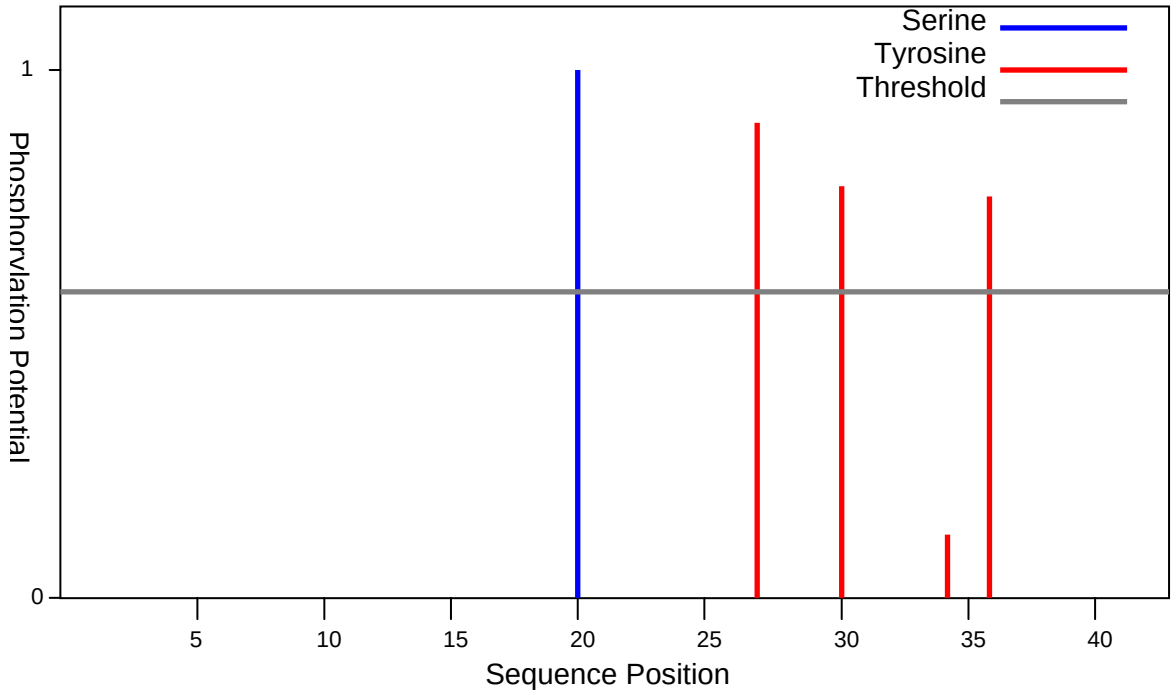

Figure 13 -NetPhos 2.0 predicted phosphorylation sites in sequence for histatin 1.

MS/MS experiments suggested that the poly-phosphorylation of histatin 1 involves the four C-terminal tyrosines. Although the score is higher for a serine phosphorylation, NetPhos gives a result for the most probable tyrosines involved in phosphorylation that is in agreement with the results of this work.

This implies the action of an unknown tyrosine kinase in the secretion pathway of histatin 1.

CONCLUSIONS

The experiments and results presented in this thesis demonstrate that mass spectrometry was a useful tool to identify the poly-phosphorylations of histatin 1.

It was demonstrated that the additional four phosphorylations occurring in histatin 1 isoforms, besides the already established on Ser-2, are located on the four tyrosines of the C-terminal: Tyr-27, Tyr-30, Tyr-34, and Tyr-36. Although the determination of all the residues that undergo phosphorylation processes was performed, it cannot be affirmed unambiguously which of the tyrosine residues are involved in the di-, tri-, or tetra-phosphorylated histatins 1.

Further experiments will be necessary to determine with exactitude the phosphorylation site on the di-, tri-, and tetra-phosphorylated histatins 1.

In future perspectives lies the characterization of the kinase responsible for the histatin 1 isoforms phosphorylations, determination of histatin 1 function, as well as the evaluation of the different histatin 1 isoforms profile in individuals, since preliminary data indicated different percentages on some pathological samples (Onnis et al., 2005).

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APPENDIX

Table 3 – Peptide fragments obtained from the trypsin digestion.

		Fragment	PHOS	Theoretical Mass (Da)	Experimental Mass (Da)	Error (Da)
Trypsin	MALDI-TOF	DSHEKRHHGYRR	0	1577.69	1577.98	0.29
		DSHEKRHHGYRR	1	1657.66	1657.83	0.17
		DSHEKRHHGYRRK	0	1705.86	1705.13	-0.73
		DSHEKRHHGYRRK	1	1785.83	1785.34	-0.49
		HHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN	3	4335.29	4336.88	1.59
		RKFHEKHHSHREFPFYGDYGSNYLYDN	0	3444.69	3444.36	-0.33
		KFHEKHHSHR	0	1342.49	1342.78	0.29
		KFHEKHHSHREFPFYGDYGSNYLYDN	0	3288.50	3289.39	0.89
		FHEKHHSHREFPFYGDYGSNYLYDN	0	3160.33	3160.70	0.37
		HHSHREFPFYGDYGSNYLYDN	0	2618.72	2618.92	0.20
		EFPFYGDYGSNYLYDN	0	1964.03	1963.78	-0.25
	HPLC-ESI	DSHEK	1	694.58	695.73	1.15
		DSHEKRHHGYR	0	1421.50	1421.05	-0.45
		DSHEKRHHGYR	1	1501.47	1500.63	-0.84
		DSHEKRHHGYRR	0	1577.69	1577.81	0.12
		DSHEKRHHGYRR	1	1657.66	1656.60	-1.06
		DSHEKRHHGYRRKFHEKHHSHR	0	2902.16	2903.00	0.84
		RHHGYR	0	824.90	824.50	-0.40
		HHGYR	0	668.71	668.30	-0.41
		HHGYRR	0	824.90	824.50	-0.40
		RKFHEK	0	843.98	843.49	-0.49
		RKFHEKHHSHR	0	1498.67	1499.00	0.33
		RKFHEKHHSHREFPFYGDYGSNYLYDN	0	3444.69	3444.40	-0.29
		RKFHEKHHSHREFPFYGDYGSNYLYDN	2	3604.62	3604.20	-0.42

		KFHEK	0	687.80	687.33	-0.47
		KFHEKHHSHR	0	1342.49	1341.87	-0.62
		KFHEKHHSHREFPFYGDYGSNYLYDN	0	3288.50	3287.80	-0.70
		KFHEKHHSHREFPFYGDYGSNYLYDN	1	3368.47	3368.20	-0.27
		KFHEKHHSHREFPFYGDYGSNYLYDN	2	3448.43	3448.40	-0.03
		KFHEKHHSHREFPFYGDYGSNYLYDN	3	3528.40	3528.30	-0.10
		FHEK	0	559.62	559.30	-0.32
		HHSHREFPFYGDYGSNYLYDN	0	2618.72	2618.10	-0.62
		EFPFYGDYGSNYLYDN	0	1964.03	1963.72	-0.31

Table 4 – Peptide fragments obtained from the Proteinase K digestion.

		Fragment	PHOS	Theoretical Mass (Da)	Experimental Mass (Da)	Error (Da)
Proteinase K	MALDI-TOF	DSHEKRHHGYRRKF	1	1933.01	1933.12	0.11
		HEKRHHGYRRKFHEKHHSHREFPFY	1	3463.72	3465.73	2.01
		RRKFHEKHHSHREF	0	1931.15	1932.28	1.13
		HEKHHSHREFPFY	1	1830.87	1830.29	-0.58
		HEKHHSHREFPFYGDY	2	2246.14	2247.21	1.07
		HEKHHSHREFPFYGDYGSNY	0	2507.62	2508.06	0.44
		HREFPFYGDY	0	1330.42	1330.84	0.42
		HREFPFYGDYGS	1	1554.52	1554.12	-0.40
		HREFPFYGDYGSNY	1	1831.80	1831.15	-0.65
		HREFPFYGDYGSNYL	1	1944.96	1945.11	0.15
		HREFPFYGDYGSNYLY	0	2028.17	2029.30	1.13
		PFYGDY	2	920.73	919.60	-1.13
		PFYGDYGSNYL	1	1375.34	1375.11	-0.23
		PFYGDYGSNYLY	1	1538.52	1538.14	-0.38
		PFYGDYGSNYLYDN	1	1767.71	1766.22	-1.49
		YGDYGSNYLYDN	3	934.67	933.69	-0.98
		GDYGSNYLYDN	1	1360.24	1361.10	0.86
	HPLC-ESI	RRKFHEKHHSHREF	0	605.74	605.45	-0.29
		HEKHHSHREFPFY	1	1830.87	1830.15	-0.72
		HREFPF	0	831.93	831.51	-0.42
		HREFPFY	0	995.11	995.47	0.36
		HREFPFY	1	1075.08	1075.60	0.52
		YGDY	0	516.51	516.10	-0.41
		YGDY	2	676.44	676.44	0.00

		GSNY	0	439.43	439.10	-0.33
		GSNYL	0	552.58	552.20	-0.38
		GSNYLY	0	715.76	716.33	0.57
		NYLYDN	1	880.79	881.39	0.60
		LYDN	0	523.54	523.10	-0.44

Table 5 – Peptide fragments obtained from the Protease V8 digestion.

		Fragment	PHOS	Theoretical Mass (Da)	Experimental Mass (Da)	Error (Da)
Protease v8	MALDI-TOF	DSHEKRHHGYRRKFHE	1	2198.01	2199.27	1.26
		KRHHGYRRKFHE	0	1649.88	1650.79	0.91
		KRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYD	0	4263.02	4263.28	0.26
		KHHSHREFPFYGD	0	1655.76	1656.61	0.85
		KHHSHREFPFYGDYGSNYLYD	2	2791.09	2790.61	-0.48
		FPFYGDYGSNYLYDN	0	1833.75	1834.44	0.69
		YGSNYLYDN	3	1347.35	1347.06	-0.29
	HPLC-ESI	DSHEKRHHGYRRKFHE	0	2118.01	2119.23	1.22
		DSHEKRHHGYRRKFHE	1	2198.01	2199.25	1.24
		SHE	0	371.14	371.20	0.06
		KRHHGYRRKFHE	0	1649.88	1650.17	0.29
		KRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYD	0	4263.02	4262.40	-0.62
		KRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYD	3	4502.92	4504.0	1.08
		KHHSHREFPFYGDYGSNYLYDN	0	2745.20	2746.40	1.20
		KHHSHREFPFYGDYGSNYLYDN	1	2825.17	2826.40	1.23
		KHHSHREFPFYGDYGSNYLYDN	2	2905.13	2905.80	0.67
		KHHSHREFPFYGDYGSNYLYDN	3	2985.10	2985.80	0.70
		KHHSHREFPFYGDYGSNYLYDN	4	3065.07	3065.80	0.74
		FPFYGD	0	744.31	744.50	0.19
		FPFYGDYGSNYLYDN	0	1833.75	1833.61	-0.14
		YGSNYLYDN	0	1107.45	1107.39	-0.06